

AMENDMENTS TO THE SPECIFICATION:

On page 1, please delete the paragraph at lines 4-7, and replace with the following:

This application is a Division of U.S. Application Serial No. 09/771,035, filed January 25, 2001, which is a Continuation-in-Part of PCT/US00/20261, filed July 21, 2000; which is a Continuation-in-Part of U.S. Application Serial No. 09/232,170, filed on January 15, 1999; which is a Continuation-In-Part of U.S. Application Serial No. 09/008,186, filed on January 16, 1998. All the above applications are incorporated herein by reference.

Page 1, lines 13-20:

Great interest exists in launching genome projects in human and non-human genome project. The human genome has between 2.8 million and 3.5 million base pairs, about 3 percent of which are made of genes. In June 2000, the Human Genome Project and biotech company Celera Genomics announced that a rough draft of the human genome has been completed (~~<http://www.ncbi.nlm.nih.gov>~~see National Center for Biotechnology Information (NCBI) database website). This information, however, will only represent a reference sequence of the human genome. The remaining task lies in the determination of sequence functions, which are important for the study, diagnosis, and treatment of human diseases.

Page 1, lines 21-25:

The Mouse genome is also being sequenced. Genbank provides about 1.2% of the 3-billion-base mouse genome (~~<http://www.informatics.jax.org>~~see Mouse Genome Informatics (MGI) database website) and a rough draft of the mouse genome is expected to be available by 2003 and a finished genome by 2005. In addition, the *Drosophila* Genome Project has recently been completely sequenced (~~<http://www.fruitfly.org>~~see Berkeley Drosophila Genome Project database website).

Page 6, lines 1- 10:

MacFarlane and Popovich (Virology 267:29-35 (2000)) constructed viral vectors from infectious cDNA clones of each of the three tobnaviruses, tobacco rattle virus (TRV), pea early-browning virus (PEBV), and pepper ringspot virus (PepRSV). RNA2 of each of the three viruses was modified to carry an additional coat protein subgenomic promoter and was used to express green fluorescent protein (GFP). The TRV-GFP construct was prepared by removal of 3' part of the 2b gene and the entire 2c gene. The PEBV-GFP construct was prepared by removal of 2b and 2c genes. The PepRSV-GFP was prepared by removal of 3' part of the 2b gene and the entire 2c gene. ~~[is this correct?]~~ The modified RNA2 constructs that MacFarlane and Popovich teach do not have the entire 2b gene.

Page 7, line 24:

FIGS. 3A-3D lists the DNA sequence of the TRV RNA-1 clone pLSB-1 (SEQ ID NO: 1).

Page 9, lines 3-4:

FIGS. 15A-15B depicts the nucleotide sequence comparison of *A. thaliana* 740 AT #120 and *O. sativa* est D17760. (SEQ ID NOS: 11 and 12).

Page 15, line 25 to page 16, line 21:

TRV is able to infect a wide range of plant hosts, including *Arabidopsis thaliana*, *Nicotiana* species, *Brassica campestris*, *Capsicum annuum*, *Chenopodium amaranticolor*, *Glycine max*, *Lycopersicon esculentum*, *Narcissus pseudonarcissus*, *Petunia X hybrida*, *Pisum sativum*, *Solanum tuberosum*, *Spinacia oleracea*, *Vicia faba*, (<http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdb/72010004.htm#SymptHosts>) see web page for The Universal Virus Database of the International Committee on Taxonomy of Viruses (ICTVdB). TRV RNA 1 (**FIGURE 1A**) encodes proteins involved in viral replication (Replicase, 134/194 kDa) and movement (Movement Protein (MP) 29 kDa), as well as Cysteine Rich Protein (CRP,

16 kDa). TRV RNA-2a (**FIGURE 1B**) encodes 22 kDa coat protein that encapsidates RNA-1 and RNA-2 separately into rod shaped particles. The 2b gene encodes for a 40 kDa protein that is required for nematode transmissibility. The 2c gene encodes a 33 kDa protein whose function is not known (MacFarlane, *J Gen. Virol.* 80:2799-807 (1999)). In one embodiment of the invention, the invention provides a monopartite RNA viral vector derived from a tobnavirus. The monopartite RNA viral vector comprises tobnavirus RNA-1 that comprises an inserted foreign RNA sequence coding for all or part of a protein. The foreign RNA sequence can be inserted any place in RNA-1 as long as the insertion does not affect the replication and infectivity of the viral vector. For example, the foreign RNA sequence can be inserted upstream or downstream of the RNA sequence encoding a 16k Da cysteine-rich protein of RNA-1. In a preferred embodiment, the inserted foreign RNA sequence is operably linked to the 3'end of the stop codon of the RNA sequence which codes for a 16k Da cysteine-rich protein of RNA- 1. Currently, there are no published reports of multipartite viruses being modified for use as a monopartite silencing system. A monopartite silencing system would be useful for high throughput genomics screening whereby thousands of hosts are inoculated with a virus containing a library of different genes. Viral induced gene silencing of the gene library would create host gene knockouts. Having one DNA template to use for transcribing infectious RNA instead of two would simplify the process of creating gene knockouts. This could make a genomics screening project using a viral vector derived from a tobnavirus more economically feasible. In addition, because the 2a (coat protein) and 2b genes from RNA-2 are required for nematode transmissibility (MacFarlane, 1999), an RNA-1 only system would be safer for outdoor field trials.

Page 40, lines 1-14:

--The next step to making a monopartite silencing vector was to insert a plant gene to silence into pLSB-1 PL at the polylinker region. Phytoene desaturase (PDS) was chosen for its distinctive visual phenotype when expression of this gene is knocked out due to viral induced gene silencing. The *N. benthamiana* PDS allele 2 cDNA was PCR amplified

from the plasmid pWPF187 containing this PDS gene (U.S. Patent No. 5,539,093, Fitzmaurice *et al.*, 1996) using the following oligonucleotides 5'-

TGGTTCTGCAGTTATGCATGCCCCAAA TTGGACTTG-3' (upstream) (SEQ ID NO: 49) and 5'- TTTTCCTTTTGC GGCCGCTAA ACTACGCTTGCTTCTG-3' (downstream) (SEQ ID NO: ~~82~~50). The 5' overhangs of these oligonucleotides contain unique *Nsi* I and *Not* I sites, which were incorporated upstream and downstream, respectively, of the PDS gene. The phytoene desaturase cDNA was then subcloned into the *Sse* 8387 I/*Not* I sites of pLSB-1 PL to make pLSB-1 PDS (+) (**FIGURE 10**). Note that *Sse* 8387 I (Amersham Pharmacia Biotech Inc., Piscataway, NJ 08855) and *Nsi* I produce compatible cohesive ends.—

Page 48-49, lines 17-36:

--A 488 bp cDNA from *N. benthamiana* stem cDNA library was isolated by polymerase chain reaction (PCR) using the following oligonucleotides: ATARFK15, 5' AAG AAG GAG ATG CGA ATT CTG ATG GT 3' (upstream) (SEQ ID NO:55), ATARFN176, 5' ATG TTG TTG GAG AGC CAG TCC AGA CC 3' (downstream) (SEQ ID NO: 56). The vent polymerase in the reaction was inactivated using phenol/chloroform, and the PCR product was directly cloned into the *Hinc*II site in Bluescript KS+ (Stratagene). The plasmid map of KS+ Nb ARF #3, which contains the *N. benthamiana* ARF ORF in pBluescript KS+ is shown in **FIGURE 16**. The nucleotide sequence of *N. benthamiana* KS+ Nb ARF#3, which contains partial ADP-ribosylation factor ORF, was determined by dideoxynucleotide sequencing. The nucleotide sequence from KS+ Nb ARF#3 had a strong similarity to other plant ADP-ribosylation factor sequences (82 to 87% identities at the nucleotide level). The nucleotide sequence comparison of *N. benthamiana* KS+ Nb ARF#3 and *A. thaliana* 740 AT #120 shows a high homology between them (**FIGURE 17**, SEQ ID NOs: ~~63~~13 and ~~64~~14 respectively). The nucleotide sequence of KS+ NbARF #3 exhibits a high degree of homology (77-87% identities and positives) to plant, yeast and mammalian DNA encoding ARFs (Table 4). Again, the high homology of DNAs encoding GTP binding proteins from yeast, plants,

human, bovine and mice indicates that DNAs from one donor organism can be transfected into another host organism and effectively silence the endogenous gene of the host organism.--

Page 54, lines 9-23:

Several clones analyzed produced a phenotype that would be predicted if the corresponding endogenous gene was inhibited or silenced. For example, for a TRV RNA2 clone containing sequence homologous to α -tubulin induced severe shoot and root stunting, as expected since α -tubulin is involved in cell division in the meristematic tissue. Inhibition of α -tubulin with herbicides in the dinitroaniline family has been shown to also result a similar phenotype in plants

(<http://www.agcom.purdue.edu/AgCom/Pubs/WS/WS-23.html> see Purdue University Cooperative Extension Work in Agriculture and Home Economics web page, *Herbicide Mode-Of-Action Summary*, Ross, et al.). An RNA-2 clone containing sequence homologous to uroporphyrinogen decarboxylase, an enzyme involved in porphyrin and chlorophyll metabolism, induced necrosis on infected leaves similar to that seen in a hypersensitive response in plants. It has been previously reported that expression of the antisense RNA for this enzyme resulted in the production of "necrotic leaf lesions" (Mock, et al. *JBiol. Chem.* 12:4231-8 (1999)). Collectively, these results strongly show the utility of TRV in silencing endogenous plant genes using homologous gene sequences. Information gained from studies in *N. benthamiana* could be extended and applied to identifying novel genes in *Arabidopsis*.

Page 56, lines 14-28:

--The 5-enolpyruvyl-shikimate-3 phosphate synthase (EPSPS) gene encodes an enzyme involved in the conversion of shikimic acid to chorismic acid and is the enzyme target of the herbicide Roundup[®]. The EPSPS gene from *N. tabacum* was PCR amplified from the plasmid 736.1 using the following oligonucleotides: 5'- TGGTTCTGCAG TTATGCATGGCACAGATTAGCAGCATG-3' (upstream) (SEQ ID NO: 57) and 5'-

GGTACCAAGCTTGCGGCCGCTTAATGCTTGGAGTACTCCTG-3' (downstream)
(SEQ ID NO:58). This PCR product was subcloned into pK20-2b-P/N-SmaI in the positive orientation to result in the construct pK20-2b-EPSPS(+). **(Figure 19)** When transcripts from pK20-2b-EPSPS(+) were coinoculated with pLSB-1 transcripts onto *N. benthamiana*, chlorotic patches on the systemic leaves were observed after 10-14 days. Samples from infected plants were analyzed using GC/MS. GC/MS analysis revealed that there was a 47-fold increase in shikimic acid accumulation compared to the TRV-GFP control, as expected from a positive sense inhibition of the EPSPS enzyme and an accumulation of the enzyme substrate. Plants treated with Roundup[®] showed a 69-fold increase in shikimic acid. –

Page 58, lines 7-20:

One to two weeks after inoculation, transfected *Nicotiana benthamiana* plants were visually monitored for changes in growth rates, morphology, and color. One set of plants transfected with pK20-D 11(1) (FIGURE 22) were stunted and had necrotic leaves. DNA sequence analysis (FIGURE 23, SEQ ID NO: 18) revealed that this clone contained a *Nicotiana benthamiana* DEAD box RNA helicase open reading frame (ORF) in the positive orientation. The pK20-D 11(1) encoded protein sequence exhibited a high degree of homology (70-74% and 82-85%, identities and positives, respectively) to yeast, insect and human DEAD box RNA helicase proteins (Table 6). The DEAD box RNA helicase family containing the highly conserved residues, Asp-Glu-Ala-Asp, are involved in diverse biological functions such as ribosome assembly, translation initiation, and RNA splicing. They modulate regulatory factors during organ maturation, cell growth and differentiation. Although the DEAD box RNA helicase family has been described in *Arabidopsis thaliana* (NAR review), the actual function of the genes encoding "putative 20 computer-predicted helicases" has not been determined experimentally.